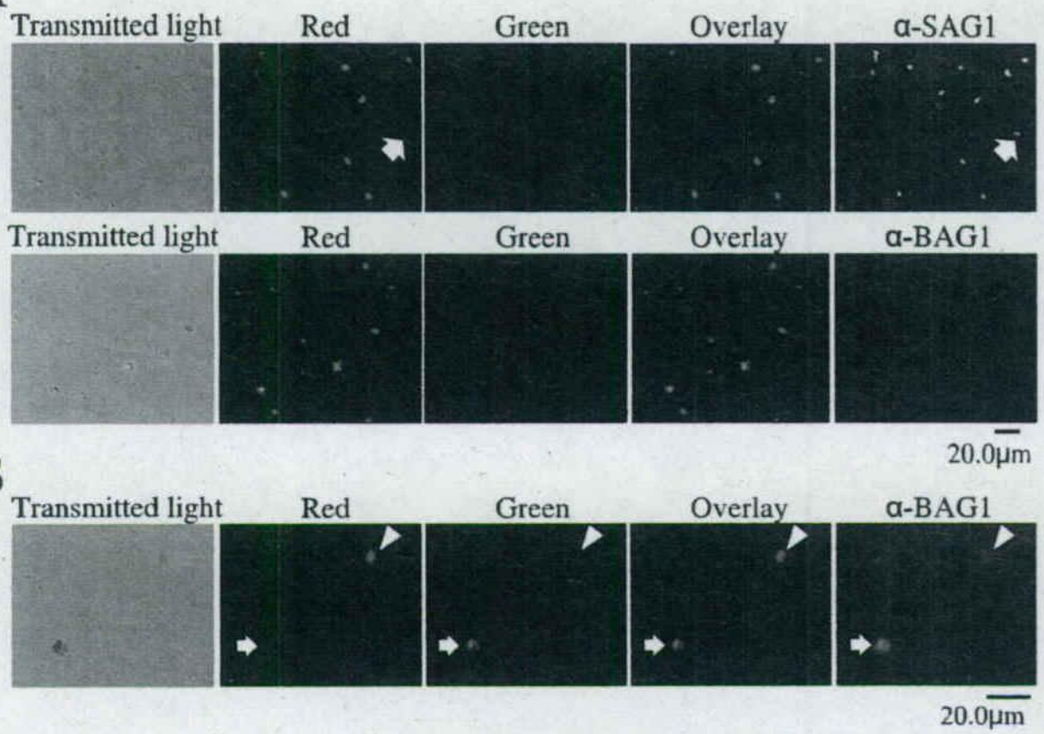
Fig. 2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B
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Fig. 3

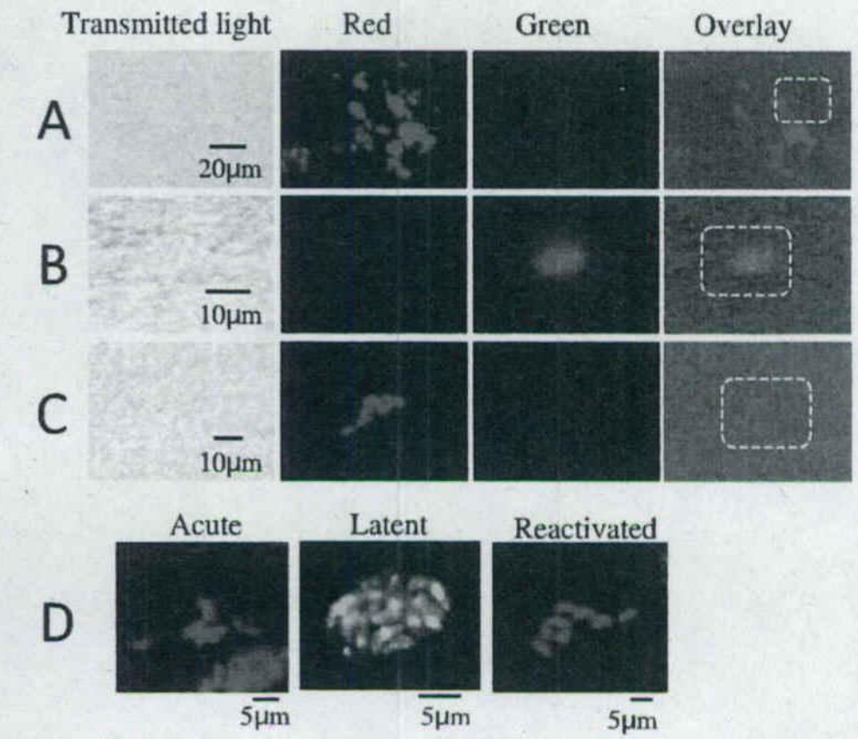
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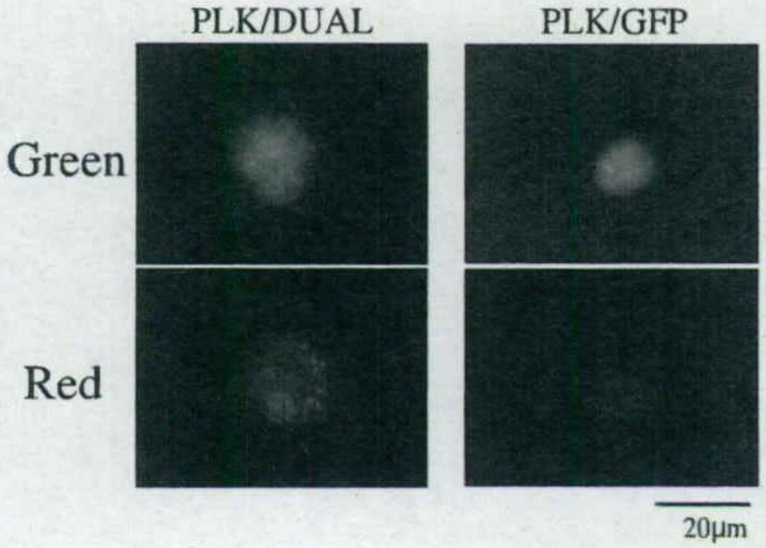
Fig. 4



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Short communication

Dissemination of extracellular and intracellular *Toxoplasma gondii* tachyzoites in the blood flow

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ABSTRACT

Toxoplasma gondii is an intracellular parasite. It has been thought that *T. gondii* can disseminate throughout the body by circulation of tachyzoite-infected leukocytes (intracellular parasite) in the blood flow. However, a small number of parasites exist as free extracellular tachyzoites in the blood flow (extracellular parasite). It is still controversial whether the extracellular parasites in the blood flow disseminate into the peripheral tissues. In this study, we evaluated the dissemination efficiency of the extracellular and intracellular parasites in the blood flow using GFP-expressing transgenic parasite (PLK/GFP) and DsRed Express-expressing transgenic parasite (PLK/RED). When PLK/GFP and PLK/RED tachyzoites were injected, as intracellular and extracellular forms respectively, at the same time into the tail vein of a mouse, many disseminated green fluorescent PLK/GFP tachyzoites were observed in the lung, the spleen, the liver and the brain. However, only a few red fluorescent PLK/RED tachyzoites were detected in these organs. When PLK/GFP and PLK/RED tachyzoites were injected in the opposite manner, that is, as extracellular and intracellular forms respectively, the majority of tachyzoites in these tissues were PLK/RED tachyzoites. Collectively, these results indicate that intracellular tachyzoites mainly disseminate throughout the body and that extracellular tachyzoites hardly contribute to parasite dissemination.

Toxoplasma gondii is an apicomplexan parasite that causes congenital infection and abortion as well as fatal infection in immune-compromised individuals. Infection generally occurs from oral ingestion of oocysts or tissue cysts. After oral ingestion, *T. gondii* initially crosses the intestinal epithelium and invades the general circulation. It was reported that *T. gondii* organisms circulate in the blood only 1 h after infection [1,2]. In the blood flow, the majority of parasites exist intracellularly, inside infected leukocytes [1–3] (Fig. 1A). The sera of several hosts, including humans, seem to have a lethal effect on extracellular tachyzoites in the absence of specific antibodies [4,5]. To escape from the lethal effect of serum, it might be necessary for *T. gondii* to infect leukocytes and be separated from serum. Recently, it was reported that infected CD11b+ leukocytes transport intracellular *T. gondii* into the brain extravascular space [3]. In this study, *T. gondii* DNA was not detected in the brain after intravenous injection of 10 or 50 extracellular tachyzoites [3]. It is suspected that trafficking of leukocytes contribute to dissemination of intracellular parasites in a 'Trojan horse' mechanism. However, despite these reports, a small number of extracellular parasites were observed in the blood flow [3] (Fig. 1A). It is also reported that *T. gondii* tachyzoites are resistant to human complement in the absence of specific antibodies [6,7]. An *in vivo* study using IgM−/− mice also showed that natural IgM does not function to limit parasite dissemination in the absence of specific antibodies against *T. gondii* [8]. These reports suggest the possibility that extracellular parasites survive in the blood flow and contribute to the dissemination to the peripheral tissues. The contribution of extracellular parasites to *T. gondii* dissemination to the peripheral tissues is controversial. In this study, we investigated disseminations of extracellular and intracellular tachyzoites in the blood flow. All experiments using animals were performed in accordance with the Gifu University Animal Care and Use Committee guidelines.

To compare the abilities of the intracellular and extracellular tachyzoites in the blood flow to disseminate into the peripheral tissues, we designed an experiment using PLK strain (Type II) [9] derived transgenic *T. gondii* tachyzoites stably expressing green and red fluorescence, PLK/GFP and PLK/RED, respectively [10–12]. In this experiment, PLK/GFP and PLK/RED tachyzoites are directly injected into the blood flow of one mouse in intracellular and extracellular forms, at the same time. Then, disseminations of both parasites were chased *in vivo* by detection of green and red fluorescent parasite in mouse tissues.

The tachyzoites of PLK/GFP and PLK/RED were passaged in Vero cells maintained in RPMI-1640 medium supplemented with 7.5% fetal

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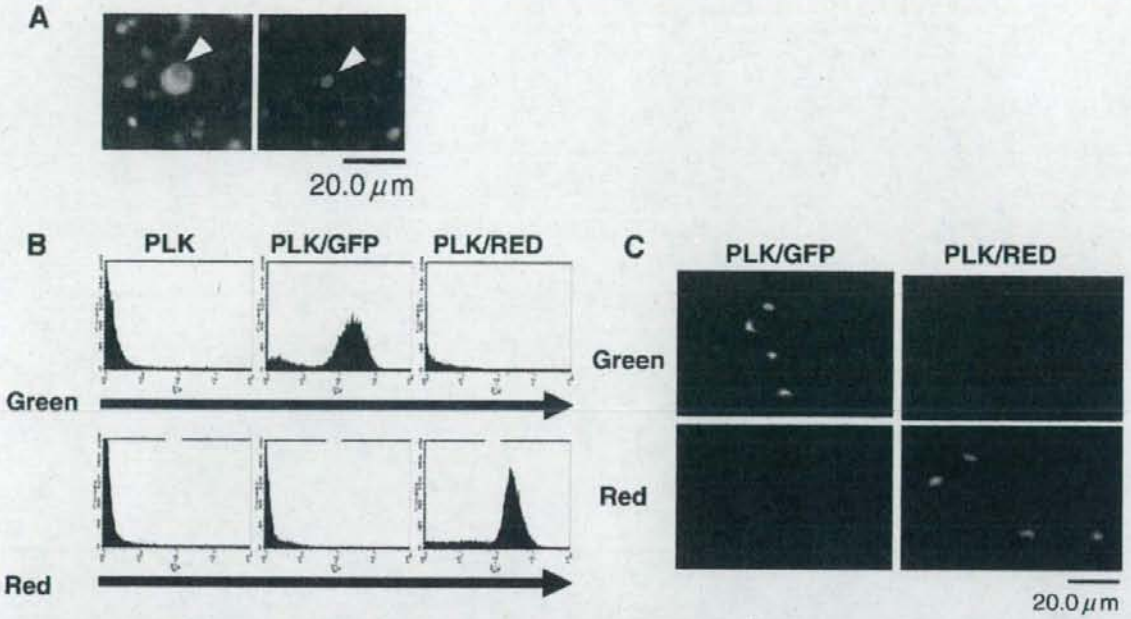

Fig. 1. Fluorescence of PLK/GFP and PLK/RED tachyzoites. (A) Intracellular and extracellular tachyzoites. To facilitate the confirmation of intracellular and extracellular tachyzoites in the blood flow, GFP-expressing transgenic mice (C57BL/6-Tg(CAG-EGFP) C14-Y01-FM131 Osb) [13,14] was i.p. infected with 10^6 red fluorescent *T. gondii* (PLK/RED) tachyzoites. The peripheral blood samples were observed 4 days p.i. Left and right panels show PLK/RED tachyzoite in a leukocyte expressing GFP (intracellular tachyzoite) and PLK/RED tachyzoite outside leukocytes (extracellular tachyzoite), respectively. (B) Fluorescence intensity of PLK, PLK/GFP and PLK/RED tachyzoites (left, center and right, respectively). Upper panels show green fluorescence detected by the FL-1 channel; lower panels show red fluorescence detected by the FL-2 channel. (C) Extracellular tachyzoites of PLK/GFP (left) and PLK/RED (right) after the purification procedure. Scale bar = 20.0 μm.

calf serum (FCS) and 20 μg/ml gentamicin and incubated at 37 °C in a 5% CO_2 incubator. To confirm whether PLK/GFP and PLK/RED are distinguished by fluorescence, these transgenic parasites were observed using flow cytometry and fluorescent microscope. Flow cytometry was performed on a FACSCalibur system (BD Biosciences Pharmingen, San Diego, CA) using the single tachyzoite suspension. As shown in Fig. 1B, green fluorescence from PLK/GFP was detected in the green fluorescent (FL-1) channel (upper-center panel). Leakage into the red fluorescent (FL-2) channel, which may interfere with clear distinction of PLK/GFP from PLK/RED, was not detected (Fig. 1B, lower-center panel). Similarly, PLK/RED showed red fluorescence detected in the FL-2 channel, but did not show leakage into the FL-1 channel (Fig. 1B, upper-right and lower-right). In the case of observations using a fluorescent microscope, slight leakage of fluorescence from PLK/GFP into the red channel was detected (Fig. 1C). However, the weak red fluorescence from PLK/GFP did not interfere with the ability to distinguish PLK/GFP and PLK/RED, because PLK/RED showed much brighter red fluorescence than PLK/GFP, and green fluorescence from PLK/RED was under the detectable level (Fig. 1C). These results indicate that PLK/GFP and PLK/RED tachyzoites can be distinguished from each other based on their fluorescence. Therefore we prepared the intracellular and extracellular tachyzoites using PLK/GFP and PLK/RED.

Extracellular tachyzoites were released from infected Vero cells by rapid extrusion through a 27-gauge needle, 3 times. After centrifugations at 2000 rpm for 10 min at room temperature, the pellet was re-suspended in 2 ml of phosphate-buffered saline (PBS). Cell debris was removed by filtration through a filter with a pore size of 5 μm (Millipore, Bedford, MA). The purified extracellular tachyzoites were suspended in PBS at a concentration of 2×10^4 tachyzoites/ml. To estimate the damage to parasites caused by the purification procedure, the numbers of tachyzoites conserving infectivity among 1000 obtained tachyzoites were measured as follows. The obtained tachyzoites were re-suspended in RPMI-1640 medium supplemented with 7.5% FCS and 20 μg/ml gentamicin. Extracellular tachyzoites were added to wells of the 24-well plates (10^3 tachyzoites/300 μl RPMI medium/well) and plates were incubated for 8 h at 37 °C in a 5% CO_2 incubator. After incubation for 8 h, Vero cells were washed twice with 300 μl of RPMI medium and the media were substituted by Eagle medium containing 0.5% methylcellulose supplemented with 7.5% FCS, 2 mM L-glutamine and 0.15% $NaHCO_3$ (pH 7.4). Within 16 h of the medium replacement, the numbers of PLK/GFP and PLK/RED tachyzoite clusters were counted. Each cluster contained from one to four parasites (data not shown). The experiments were independently repeated six times and the 95% confidence intervals [CIs] of the population means for the numbers of infectious tachyzoites were estimated. The numbers of infectious PLK/GFP and PLK/RED tachyzoites were 744.5±38.1 (95%CI: 649.8 to 839.2) and 679.0±145.6 (95%CI: 526.2 to 831.8), respectively.

To prepare intracellular tachyzoites, female 6–8 week-old C57BL/6J mice (purchased from Oriental Yeast Co., Ltd., Tokyo, Japan) were i.p. infected with 10^6 PLK/GFP or PLK/RED tachyzoites. Four days after the injection, the blood was collected and erythrocytes were lysed using NH_4Cl solution (0.145 M NH_4Cl, 17 mM Tris–HCl pH 7.65). After centrifugations at 1500 rpm for 5 min at room temperature to pellet leukocytes, pellets were washed twice with 10 ml of PBS and re-suspended in PBS. The obtained peripheral leukocytes were used as intracellular tachyzoite samples. The proportions of infected leukocytes were determined by observing more than 200 leukocytes using a fluorescent microscope. 1.48% and 2.91% of leukocytes were infected with PLK/GFP and PLK/RED, respectively. Although the majority of infected cells harbored a single parasite, a small number of cells containing more than two tachyzoites were also detected (data not

shown). The leukocyte suspensions were diluted in PBS to adjust the concentration of tachyzoites to 1×10^4 tachyzoites/ml. In this study, in order to facilitate the collection of enough number of infected leukocytes (intracellular tachyzoites), mice were infected i.p. with huge number of *T. gondii* tachyzoites (10^6 tachyzoites). It is not the route of natural infection of *T. gondii*. Therefore, from the i.p. inoculation to the appearance of parasites in the blood flow, dissemination route of the inoculated parasites is different from that of naturally infected ones. However, the difference would not have an impact on conclusions of this study because the subject of this study is phenomenon after the appearance of parasites in the blood flow.

Equal volumes (1:1) of the suspension of leukocytes infected with PLK/GFP and the suspension of extracellular PLK/RED tachyzoites were mixed. 200 μl of the mixed suspension was injected into the tail veins of each mouse (containing 1000 intracellular PLK/GFP and 2000 extracellular PLK/RED). A mixed suspension of extracellular PLK/GFP and leukocytes infected with PLK/RED was also prepared and injected in the same manner. Considering the survival rate of extracellular tachyzoites after the purification procedure, it appeared that approximately 1400 extracellular tachyzoites conserved their infection ability among the injected 2000 tachyzoites. Therefore, in this experimental design, approximately 1400 (little more than 1000) extracellular and 1000 intracellular infectious tachyzoites invaded the blood flow. Nine days after the injection, a huge number of disseminated PLK/RED tachyzoites were detected in the brain, the spleen, the liver and the lungs of mice infected with extracellular PLK/GFP and intracellular PLK/RED (Fig. 2A and C). However, only a few PLK/GFP tachyzoites were also detected in these organs (Fig. 2A and C). When PLK/GFP and PLK/RED tachyzoites were injected in the opposite combination, that is, intracellular PLK/GFP and extracellular PLK/RED, a huge number of PLK/GFP tachyzoites disseminated into these organs (Fig. 2B and C). PLK/RED tachyzoite was not detected in these organs, except for two PLK/RED tachyzoites detected in the lungs (Fig. 2C). The several preliminary experiments also showed the same tendency (data not shown). It is impossible to eliminate the possibility that the proliferation speeds of the two transgenic parasites, PLK/GFP and PLK/RED, are slightly different, and that this affected the experiment results. However, such an effect of proliferation speed would not have an impact on the conclusion of the experiments, because the massive majority of disseminated parasites in the tissues were intracellular tachyzoites, in both combinations of injection (extracellular PLK/GFP and intracellular PLK/RED; extracellular PLK/RED and intracellular PLK/GFP) (Fig. 2). These results indicate that intracellular tachyzoites mainly disseminate throughout the body and that extracellular tachyzoites hardly contribute to dissemination.

In our experiments, only a few parasites that had been administered into the blood flow as an extracellular form were detected in tissues (Fig. 2). These data suggest that the majority of extracellular tachyzoites could not arrive at the peripheral tissue. When we injected 10^6 extracellular PLK/RED tachyzoites into the tail vein of GFP-expressing transgenic mouse (C57BL/6-Tg(CAG-EGFP) C14-Y01-FM131 Osb) [13,14] and collected the peripheral blood after 1 h, the number

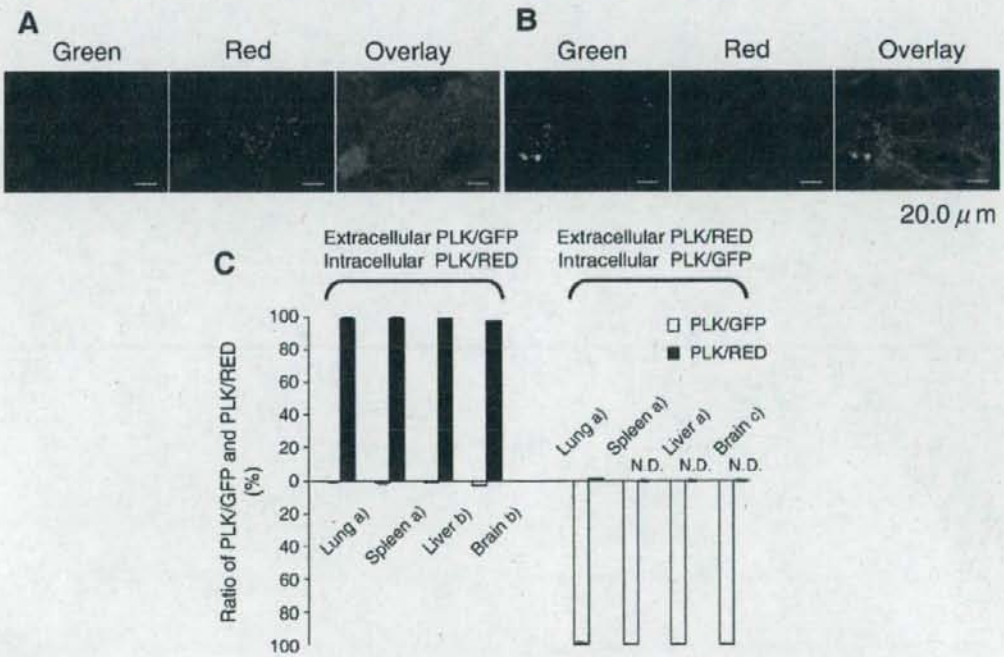

Fig. 2. Dissemination of extracellular and intracellular tachyzoites *in vivo*. (A) Half of each lung of mice infected with extracellular PLK/GFP and intracellular PLK/RED tachyzoites was fixed in 4% paraformaldehyde and embedded in plastic resin (Technovit 8100, Heraeus Kulzer, Wehrheim, Germany) according to the manufacturer's instructions. Thin sections of the embedded organs were directly observed using a fluorescent microscope. Results shown are representative of three mice in the group. Scale bar = 20.0 μm. (B) The lung of mouse infected with extracellular PLK/RED and intracellular PLK/GFP tachyzoites was observed as described above. Scale bar = 20.0 μm. (C) Ratios of PLK/GFP and PLK/RED tachyzoites in the lung, the spleen, the liver and the brain of mice infected with extracellular PLK/GFP and intracellular PLK/RED tachyzoites (left), and with extracellular PLK/RED and intracellular PLK/GFP tachyzoites (right). The remaining half of lungs and other organs were chopped up into small pieces, crushed onto glass slides and observed using a fluorescent microscope. The numbers of detected PLK/GFP and PLK/RED tachyzoites were determined. Except for some organ samples in which only a few tachyzoites were detected (shown as superior letter 'b' and 'c'), in total, more than 200 (the lung) or 100 (the brain, the liver and the spleen) tachyzoites were observed in each organ. Open and solid bars show levels of PLK/GFP and PLK/RED, respectively. N.D. means non-detection. a) Values are means ± SD ($n = 3$). b) Means of data from 2 mice are shown because only a few tachyzoites (all detected tachyzoites were PLK/RED) were detected in the other mouse, in spite of more than 200 fields of view being observed (×400). c) Data of one mouse are shown because only a few tachyzoites (all detected tachyzoites were PLK/GFP) were detected in the other two mice, in spite of more than 200 fields of view being observed (×400).

of extracellular tachyzoites (red tachyzoites outside green leukocyte), in the blood sample was under detectable level (data not shown). It appears that extracellular tachyzoites were immediately eliminated by innate immunity after the direct exposure to serum.

Recently, it was reported that infected CD11c- and CD11b-expressing leukocytes pass through the blood–brain barrier and transport intracellular *T. gondii* into the brain [3]. In this report, extracellular tachyzoites inoculated into mice via the tail vein were unable to pass through the blood–brain barrier and invade the brain [3]. By contrast, we detected a few PLK/GFP tachyzoites, which had been inoculated via the tail vein as the extracellular form, in the brain (Fig. 2C). It is impossible to eliminate the possibility that extracellular tachyzoites are able to pass through the brain–blood barrier at a low efficiency, and that the low level invasion of extracellular tachyzoites into the brain was missed in previous studies. However, it is likely that the inoculated extracellular PLK/GFP tachyzoites infected leukocytes in other organs (for example, the spleen, liver and lymph nodes) or in the blood vessel and then passed through the blood–brain barrier as an intracellular form, because we detected PLK/GFP tachyzoites not only in the brain, but also in the liver and the spleen on the 9th day after inoculation (Fig. 2). As shown in Fig. 2C, although greatly outnumbered, extracellular tachyzoites were also able to disseminate. Previously, it was also suggested that the gliding motility of extracellular parasites was involved in their crossing of biological barriers [15,16]. In spite of the killing effect of serum [4,5], extracellular tachyzoites may survive short-term exposure to serum. Taken together, these findings suggest that infected leukocytes transport tachyzoites into the peripheral tissues and that the infected leukocytes migrate into parenchyma as a "Trojan horse"horse", and/or transported intracellular tachyzoites egress from leukocytes in the microvasculature of peripheral tissues and invade into parenchyma as extracellular forms during a short period of time. Regardless, transportation by infected leukocytes seems to be important for dissemination of *T. gondii*. To examine whether extracellular tachyzoites can migrate into leukocytes in the blood flow, 10^6 extracellular PLK/RED tachyzoites were injected into the tail veins of GFP-expressing transgenic mice [13,14], and peripheral leukocytes were observed after 1 h. However, green cells harboring red PLK/RED tachyzoites, like that shown in Fig. 1A, were not observed (data not shown). This result indicates that invasion of extracellular parasites into leukocytes does not occur, or occurs at low frequency in the blood flow. Infection of tachyzoites into leukocytes may mainly occur not in the blood flow, but in the lymphoid organs.

Our study demonstrates that intracellular tachyzoites in the blood flow are the major contributing factor to dissemination of *T. gondii* from the general circulation into the peripheral tissues. This indicates that to understand the mechanism by which *T. gondii* is disseminated into the peripheral tissues, further study of the behaviors of leukocytes in the blood flow harboring *T. gondii* is necessary.

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References

[1] Chinchilla M, Guerrero OM, Catarinella G, Reyes L. Natural and induced dissemination of *Toxoplasma gondii*: experimental model in white mice and hamsters. Rev Biol Trop 1993;41(2):197–202.
[2] Guerrero OM, Chinchilla M. *Toxoplasma gondii* (Eucoccidia: Sarcocystidae) dissemination pattern in rats after oral infection with oocysts of an avirulent strain. Rev Biol Trop 1997;44–45:131–5.
[3] Courret N, Darche S, Sonigo P, Milon G, Buzoni-Gâtel D, Tardieux I. CD11c- and CD11b-expressing mouse leukocytes transport single *Toxoplasma gondii* tachyzoites to the brain. Blood 2006;107(1):309–16.
[4] Sabin AB, Feldman HA. Dyes as microchemical indicators of a new immunity phenomenon affecting a protozoon parasite (*Toxoplasma*). Science 1948;108(2815):660–3.
[5] Kaneko Y, Takashima Y, Xuan X, Igarashi I, Nagasawa H, Mikami T, et al. Natural IgM antibodies in sera from various animals but not the cat kill *Toxoplasma gondii* by activating the classical complement pathway. Parasitology 2004;128(Pt 2):123–9.
[6] Fuhrman SA, Joiner KA. *Toxoplasma gondii*: mechanism of resistance to complement-mediated killing. J Immunol 1989;142(3):940–7.
[7] Feldman HA. The relationship of *Toxoplasma* antibody activator to the serum-properdin system. Ann N Y Acad Sci 1956;66:263–7.
[8] Couper KN, Roberts CW, Brombacher F, Alexander J, Johnson LL. *Toxoplasma gondii*-specific immunoglobulin M limits parasite dissemination by preventing host cell invasion. Infect Immun 2005;73(12):8060–8.
[9] Bohne W, Hunter CA, White MW, Ferguson DJ, Gross U, Roos DS. Targeted disruption of the bradyzoite-specific gene BAG1 does not prevent tissue cyst formation in *Toxoplasma gondii*. Mol Biochem Parasitol 1998;92(2):291–301.
[10] Nishikawa Y, Zhang H, Ibrahim HM, Ui F, Ogiso A, Xuan X. Construction of *Toxoplasma gondii* bradyzoite expressing the green fluorescent protein. Parasitol Int 2008;57(2):219–22.
[11] Zhang G, Huong VT, Battur B, Zhou J, Zhang H, Liao M, et al. A heterologous prime-boost vaccination regime using DNA and a vaccinia virus, both expressing GRA4, induced protective immunity against *Toxoplasma gondii* infection in mice. Parasitology 2007;134(Pt 10):1339–46.
[12] Takashima Y, Suzuki K, Xuan X, Nishikawa Y, Unno A, Kitoh K. Detection of the initial site of *Toxoplasma gondii* reactivation in brain tissue. Int J Parasitol 2008;38:601–7.
[13] Ikawa M, Kominami K, Yoshimura Y, Tanaka K, Nishimune Y, Okabe M. A rapid and non-invasive selection of transgenic embryos before implantation using green fluorescent protein (GFP). FEBS Lett 1995;375(1–2):125–8.
[14] Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimune Y. 'Green mice' as a source of ubiquitous green cells. FEBS Lett 1997;407(3):313–9.
[15] Barragan A, Sibley LD. Transepithelial migration of *Toxoplasma gondii* is linked to parasite motility and virulence. J Exp Med 2002;195(12):1625–33.
[16] Barragan A, Sibley LD. Migration of *Toxoplasma gondii* across biological barriers. Trends Microbiol 2003;11(9):426–30.

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Detection of the initial site of *Toxoplasma gondii* reactivation in brain tissue

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Abstract

Detection of the initial site of *Toxoplasma gondii* reactivation in brain tissue is difficult because the number of latent cysts is small and reactivation is a transient event. To detect the early stage of reactivation in mouse brain tissue, we constructed a cyst-forming strain of *T. gondii* in the tachyzoite stage, specifically expressing red fluorescence. The PLK strain of *T. gondii* was stably transfected with a red fluorescent protein gene, DsRed Express, under the control of a tachyzoite-specific SAG-1 promoter and the resulting parasite was designated as PLK/RED. Tachyzoites of PLK/RED growing in Vero cells showed red fluorescence. When C57BL/6J mice were i.p. infected with tachyzoites of PLK/RED, red fluorescent tachyzoites were detected in their brains at the fourth day p.i. However, red fluorescent tachyzoites were not detected in BALB/c mice latently infected with PLK/RED, although non-fluorescent cysts were detected in their brains. After treatment of latently infected mice with dexamethasone for 1 month, the mice showed neurological symptoms. In mice with symptoms, red fluorescent tachyzoites were again detected in their brains and in other organs. To detect the initial site of reactivation, BALB/c mice latently infected with the strain were treated with dexamethasone for 3 weeks, and brains were excised before any symptoms appeared. Excised brains were examined for red fluorescence-positive sites. By a histological study of red fluorescent-positive sites, we detected a cyst containing red fluorescent zoites, which still had a PAS stain-positive cyst wall. A few red fluorescent zoites breaking away from the cyst were also observed. The stage-specific expression of fluorescent protein facilitates detection of a rare transient event and makes it possible to detect the initial site of reactivation.

Keywords: *Toxoplasma gondii*; Reactivation; Stage-conversion; Initial site; Fluorescent protein; Brain tissue

1. Introduction

Toxoplasma gondii is a common opportunistic infection associated with acquired immunodeficiency syndrome (AIDS). Reactivation of latent brain cysts in an immunosuppressed host can produce life-threatening encephalitis (Luft and Remington, 1992). Bradyzoite-tachyzoite stage conversion and cyst rupture in the brain have been considered to be the pathogenic mechanisms underlying encephalitis (Frenkel and Escajadillo, 1987). Stage conversion of *T. gondii* has been studied using immunosuppressed animals and brain tissues of AIDS patients (Vollmer et al., 1987; Pomeroy et al., 1989; Gazzinelli et al., 1992, 1993; Sumyuen et al., 1996; Reiter-Owona et al., 2000; Djurkovic-Djakovic and Milenkovic, 2001). However, detection of the initial site of reactivation in the brain has not yet been reported. This is a difficult issue to resolve, because the number of latent brain cysts is small and reactivation is a transient event. β-galactosidase-expressing *T. gondii* was reported to be a powerful tool for in situ localisation and observation of rare stages of the *T. gondii* life cycle in mice

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(Dao et al., 2002). Using this system, Dao and colleagues (2002) detected brain cysts even in very mild infections and reactivation of brain cysts in immunosuppressed mice, demonstrated by the existence of scattered blue-stained free zoites in brain tissue. However, even after this development, the initial site of reactivation in brain tissue, a rupturing cyst or a cyst containing stage converting zoites, has not yet been detected.

Stage conversion of *T. gondii* has been studied extensively in vitro (Soete et al., 1993; Bohne et al., 1998; Singh et al., 2002; Dzierszinski et al., 2004; Vanchinathan et al., 2005). Dzierszinski and colleagues (2004) used stage-specific expression of a fluorescent marker in *T. gondii* to observe the stage conversion process in vitro. However, a fluorescent marker has not been applied to the observation of parasites in brain tissue, as fluorescence would be lost during the embedding procedure for brain tissue.

In this study, we constructed a tachyzoite-stage-specific recombinant *T. gondii* tachyzoite-stage specifically expressing a red fluorescent protein, DsRed Express. Herein we describe an embedding procedure by which the fluorescence of the tachyzoite is retained. Using this system, we detected an initial site of reactivation in a mouse brain as a red fluorescent cyst.

2. Materials and methods

2.1. Mice

C57BL/6J and BALB/c mice were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). Mice were infected with parasites and their organs were excised after euthanasia by cervical dislocation. All experiments with mice were conducted according to the guidelines issued by Gifu University.

2.2. Maintenance of parasites

The African green monkey-kidney cell line Vero was cultured in RPMI1640 medium supplemented with 7.5% FBS, at 37 °C, in a 5% CO_2 incubator. Tachyzoites of the *T. gondi* PLK strain, a clonal derivative of the ME49 strain, (Bohne et al., 1998), the green fluorescent protein (GFP)-expressing stable transgenic parasite (PLK/GFP) (Nishikawa et al., 2007; Zhang et al., 2007) and the red fluorescent protein-expressing stable transgenic parasite (PLK/RED), engineered as described below, were propagated for routine use in Vero cells. PLK/GFP expresses GFP in both tachyzoite and bradyzoite stages (Nishikawa et al., 2007; Zhang et al., 2007). PLK/RED expresses DsRed Express only in the tachyzoite stage as described below.

2.3. Molecular methods

The 3′ flanking region of the dense granule protein 1 (GRA1) gene and the 5′ flanking region of the surface antigen 1 (SAG1) gene were PCR amplified from the plasmids GRAGFP and pS800-sag/cat (kindly provided by Dr. J.C. Boothroyd, Stanford University), respectively. Primers used to amplify the 3′ flanking region of the GRA1 gene were 5′-GAATTCCTGGCGAAATCAACGCACACCAA AAACTTG-3′ (forward) and 5′-GGATCCTATACAAAT AATTAATTAAGACTACGACGA-3′ (reverse), introducing BamHI and EcoRI restriction sites. Primers used to amplify the 5′ flanking region of the SAG gene were 5′-A AGCTTTTACATCCGTTGCC-3′ (forward) and 5′-GGA TCCACTCGTCAAAAAACCAGAAG-3′ (reverse), introducing *Hind*III and BamHI restriction sites. The amplified fragments were cloned into a pT7Blue T-Vector (Novagen, Darmstadt, Germany). The resulting plasmids were designated as pT7/3Gra and pT7/SAGp, respectively. The 3′ flanking region of the GRA1 gene was obtained from the plasmid pT7/3Gra by digestion with BamHI and EcoRI, and ligated with the plasmid pUC19 digested with BamHI and EcoRI. The resulting plasmid was digested with BamHI and HindIII, and ligated with the 5′ flanking region of the SAG1 gene obtained from the plasmid pT7/SAGp digested with BamHI and HindIII. The resulting plasmids were designated pUC/SAG-GRA. The gene encoding DsRed Express was obtained from the plasmid pCMV-DsRed Express (Clontech, Palo Alto, CA) by digestion with NheI and NotI, and inserted into the BamHI site of the plasmid pUC/SAG-GRA. The resulting plasmid was designated pSAG-RED. The EcoRI and HindIII fragments of pSAG-RED were inserted into the SpeI site of the plasmid pDHFR-TSc3 (Donald and Roos, 1993) (kindly provided by D.S. Roos, University of Pennsylvania). The resulting plasmid was designated pToxo-Red. The transfection of the plasmid vector pToxo-Red into PLK tachyzoites and the selection of stable transgenics were performed as described previously (Nishikawa et al., 2003). The obtained stable transgenic parasite expressing a red fluorescent protein, DsRed Express, was designated as PLK/RED.

2.4. Infection and detection of tachyzoites of T. gondii

To observe red fluorescent tachyzoites in vivo, two 6-week-old female C57BL/6J mice were infected i.p. with 10^6 tachyzoites of PLK/RED. The brains of infected mice were excised 4 days p.i. and red fluorescent PLK/RED tachyzoites were detected by whole brain observation as described in the following section (Fig. 1G).

2.5. Detection of PLK/RED zoites by whole brain observation

A whole brain in a plastic petri dish was put on the stage of an inverted fluorescence microscope, OLYMPUS CKX41 (Tokyo, Japan), and serial focal planes on the microscope Z-axis were observed to detect red fluorescent zoites. When red fluorescent images were detected, the brain was cut into two to three pieces. The selected pieces

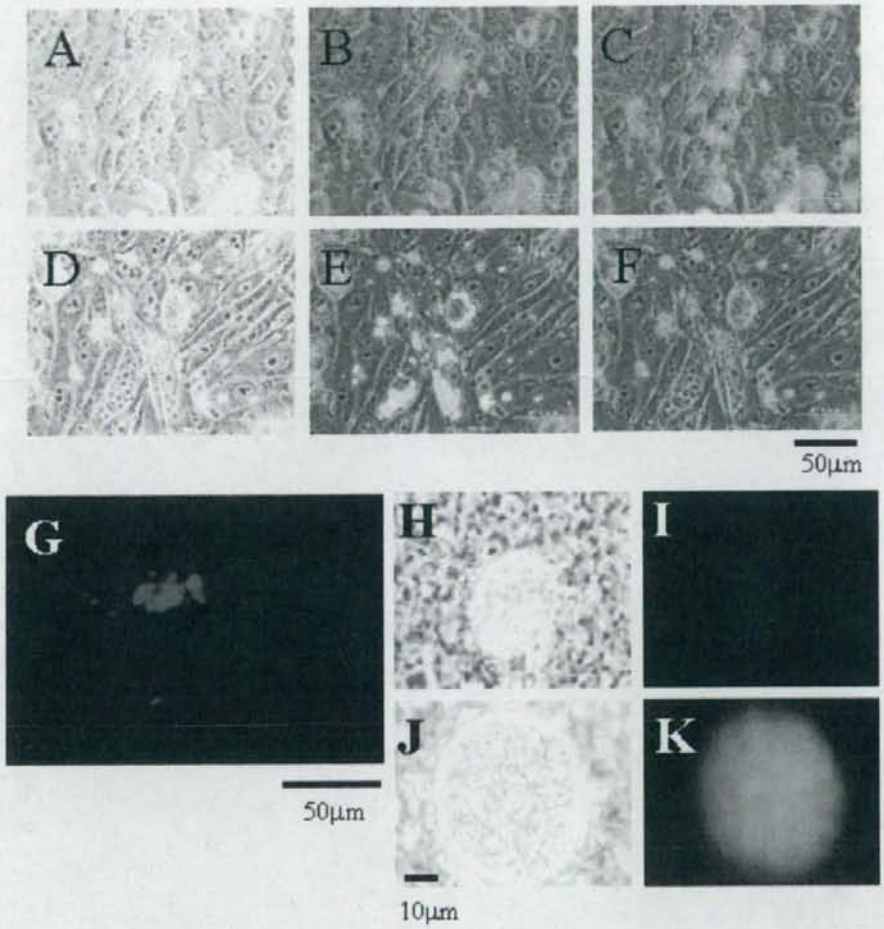

Fig. 1. Stage-specific expression of the red fluorescent protein DsRed Express. (A–F) *Toxoplasma gondii* tachyzoites, PLK, PLK/GFP and PLK/RED strains in Vero cells. Overlay of transmitted light and red fluorescence images (A–C) or green fluorescence images (D–F), respectively. (G) Red fluorescent tachyzoites of PLK/RED in C57BL/6J mouse brain. PLK/RED tachyzoites were detected by whole brain observation. (H–K) Brain cyst of PLK/RED (H and I) and PLK/GFP (J and K) in BALB/c mice. Transmitted light (H, J), red fluorescence (I) and green fluorescence (K).

containing red fluorescent zoites were used for histological analysis as described below.

2.6. Detection of latent cyst and reactivated fluorescent parasites

To observe latent cysts in mouse brain infected with PLK/GFP, two BALB/c mice (6-week-old female) were i.p. infected with 20 tachyzoites of PLK/GFP. Mice surviving more than 6 weeks without any symptoms were qualified as latently infected mice. The latently infected mice were euthanised and brains were excised. The brain samples were chopped into small pieces. The pieces of the brain were crushed onto glass slides and observed under a fluorescence microscope (Fig. 1J and K).

To count the number of latent cysts of PLK/RED, five BALB/c mice (6-week-old female) were i.p. infected with 20 tachyzoites of PLK/RED. More than 6 weeks later, brains of five mice without any symptoms were excised and chopped into small pieces. All pieces of brain tissue were crushed onto glass slides, latent cysts in the crushed pieces were observed by a fluorescence microscope and the number of cysts was counted.

To observe cyst reactivation using the PLK/RED strain, six BALB/c mice (6-week-old female) were i.p. infected with 20 tachyzoites of PLK/RED and five of these six infected mice were used for further investigation, because one mouse died on the day 37 p.i. The remaining five mice did not show any symptoms until the day 42 p.i. Therefore, we qualified the surviving mice as latently infected mice. On

day 45 p.i., one of the mice was euthanised, its brain was excised and chopped into small pieces. The pieces were crushed onto glass slides and observed under a fluorescence microscope (Fig. 1H and I). The remaining four mice were treated with dexamethasone according to the model developed previously (Djurkovic-Djakovic and Milenkovic, 2001) with minor modifications. In this study, dexamethasone (dexamethasone 21-phosphate disodium salt; Sigma) was dissolved at a concentration of 10 mg/L in the drinking water of the mice and this treatment started 52 days p.i. After 2 and 3 weeks of treatment, one mouse was euthanised at each time point and its brain was excised. Both of the excised brains were examined for red fluorescence-positive sites by whole brain observation, as described above. After whole brain observation, the brains were further investigated by histological study as follows.

The remaining two mice were continuously treated with dexamethasone. On days 34 and 39 after the start of dexamethasone treatment, each mouse showed neurological symptoms. On the day following the first observation of neurological symptoms, mice were euthanised and their brains, livers and lungs were excised. To detect red fluorescent zoites, the brains were observed by whole brain observation as described above (Fig. 2A). The livers and lungs were chopped into small pieces. These pieces were crushed onto glass slides and observed under a fluorescence microscope (Fig. 2B) or further investigated by histological analysis as described below (Fig. 2C).

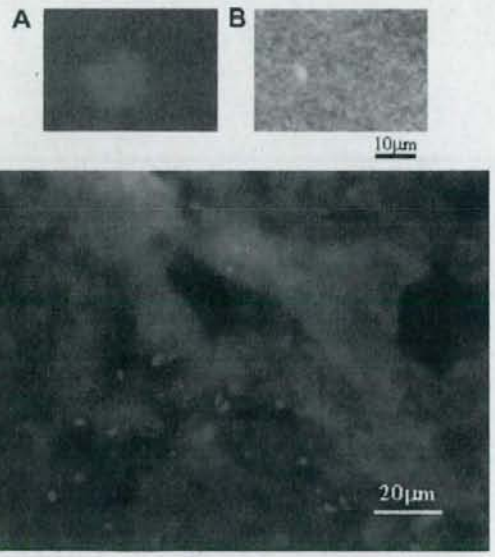

Fig. 2. Reactivated PLK/RED strain tachyzoites in brain (A), liver (B) and lung (C). After appearance of neurological symptoms, brain and liver were observed. Tachyzoites in brain, liver and lung were detected by whole brain observation, observation of a small crushed piece of tissue and observation of a thin section. (A) Red fluorescent. (B and C) Overlay of transmitted light and red fluorescent.

2.7. Histology

Small pieces of brain with red fluorescence-positive sites were fixed in 4% paraformaldehyde overnight at 4 °C. The organs were embedded in plastic resin (Technovit 8100, Heraeus Kulzer, Wehrheim, Germany) as follows. Fixed organs were washed overnight in PBS containing 6.8% sucrose at 4 °C, dehydrated in 100% acetone and embedded in plastic resin. Thin sections (3–4 μm) of the embedded organs were directly used for immunofluorescence studies or stained with H&E or PAS according to standard procedures.

3. Results

3.1. Tachyzoite-specific expression of a red fluorescent protein, DsRed Express

In order to facilitate the detection of tachyzoites in tissue, a transgenic parasite expressing a red fluorescent protein, DsRed Express, under the control of the tachyzoite-specific SAG1 promoter was established (Fig. 1A, B and C) and designated as PLK/RED. During in vitro culture for 1 month with repeated passage, loss of fluorescence was not observed. To confirm that PLK/RED tachyzoites show red fluorescence not only in vitro but also in vivo, C57BL/6J mice were infected i.p. with 10^6 PLK/RED tachyzoites. Four days p.i., whole brains were excised and directly observed under an inverted fluorescence microscope. As shown in Fig. 1G, red fluorescent tachyzoites could be detected inside brains using this method. After the detection of red fluorescence by whole brain observation, this portion was chopped into a few small pieces. The pieces of brain tissue were crushed and observed to confirm where the red fluorescent zoites existed. The fluorescent zoites shown in Fig. 1G were detected in a tissue piece carved out at a depth of about 2 mm (data not shown). Red fluorescent spots such as Fig. 1G were widely disseminated over the brain (data not shown). By contrast, the intensity of the red fluorescence of PLK/RED latent brain cysts was below the detectable threshold (Fig. 1H and I). Red fluorescent-free tachyzites were not detected in the brains of latently infected mice. As a control, tachyzoites and cysts of PLK/GFP, a PLK strain derived from recombinant *T. gondii* expressing GFP under the control of a stage non-specific promoter, were also observed. In the case of PLK/GFP, both tachyzoites and brain cysts showed bright green fluorescence (Fig. 1D, E, F, J and K).

3.2. Detection of T. gondii reactivation by red fluorescence

To observe latent cysts of PLK/RED, five brain samples were examined. The number of latent cysts was 9.4 ± 4.8 cysts/mouse and all of the detected cysts were non-fluorescent (data not shown). Red fluorescent-free tachyzoites were not detected. These results indicate that PLK/RED does not express fluorescent protein during latency.

To confirm that tachyzoites reactivated from non-fluorescent cysts express red fluorescence again, latent cysts of PLK/RED in BALB/c mouse brains were reactivated by administration of dexamethasone. On the day following the first observation of neurologic symptoms, red fluorescent tachyzoites were detected in brains, liverd, lungs (Fig. 2A, B and C) and peritoneal fluid (data not shown). The results clearly indicate that reactivated tachyzoites from non-fluorescent cysts expressed red fluorescence.

In lung tissue, red fluorescent tachyzoites were widely disseminated (Fig. 2C). On the day following the first observation, we could only harvest a small amount of peritoneal fluid with a few red fluorescent tachyzoites (data not shown). In the liver, the number of red fluorescent tachyzoites was less than in the lungs. Only a few red fluorescent tachyzoites could be detected in numerous small crushed pieces of liver (more than half the liver in total) (Fig. 2B).

Cysts containing red fluorescent zoites were not detected in brains of mice with symptoms. These results suggest that reactivation of brain cysts occurred before the appearance of clinical symptoms and that the initial site(s) of reactivation had been destroyed as reported previously (Dao et al., 2002). During latency, cysts were detected in neither livers nor lungs (data not shown). However, red fluorescent tachyzoites were detected in both livers and lungs on the day following the first observation of neurological symptoms (Fig. 2B). These observations suggest that tachyzoites reactivated in the brain reach the liver and lungs before the first appearance of symptoms.

3.3. Detection of the initial site of reactivation

To detect the initial site of reactivation, BALB/c mice latently infected with the PLK/RED strain were treated with dexamethasone. After 2 or 3 weeks of treatment, brains were excised and examined for red fluorescent zoites by whole brain observation and histological analysis. From the brain of the 3 weeks-treated mouse, only one red fluorescent spot was detected by whole brain observation. The brain was chopped into small pieces and these pieces were embedded in plastic resin, sectioned and observed as described in Section 2. As shown in Fig. 3A, B and C, a cyst containing red fluorescent zoites was detected in the piece in which red fluorescence had been detected by whole brain observation. As shown in Fig. 3E, the fluorescent cyst was detected in a granular layer of the cerebellar cortex. A few red fluorescent zoites breaking away from the cyst were also observed (Fig. 3B). The cyst still had a PAS stain-positive cyst wall (Fig. 3D). H& E staining of a thin section containing the red fluorescent cyst showed no inflammation or necrosis around the cyst (Fig. 3E). In addition to these embedded samples, half of this brain (containing both cortex and cerebra) was sectioned and all thin sections were observed. However, no other red fluorescent cysts were detected. In all thin sections from this half of the brain, 22 non-fluorescent cyst images were detected. One cyst was observed in two to four serial thin sections and it appeared that half of the brain contained five to 11 non-fluorescent cysts.

From the brain of the mouse treated for 2 weeks, neither fluorescent cysts nor fluorescent-free tachyzoites were detected by whole brain observation or histological analysis. This result suggested that in the brain of of the mouse treated for 2 week, reactivation of latent cysts had not yet occurred.

4. Discussion

We report herein a method to detect a rare and/or transient event in the *T. gondii* life cycle, using stage-specific

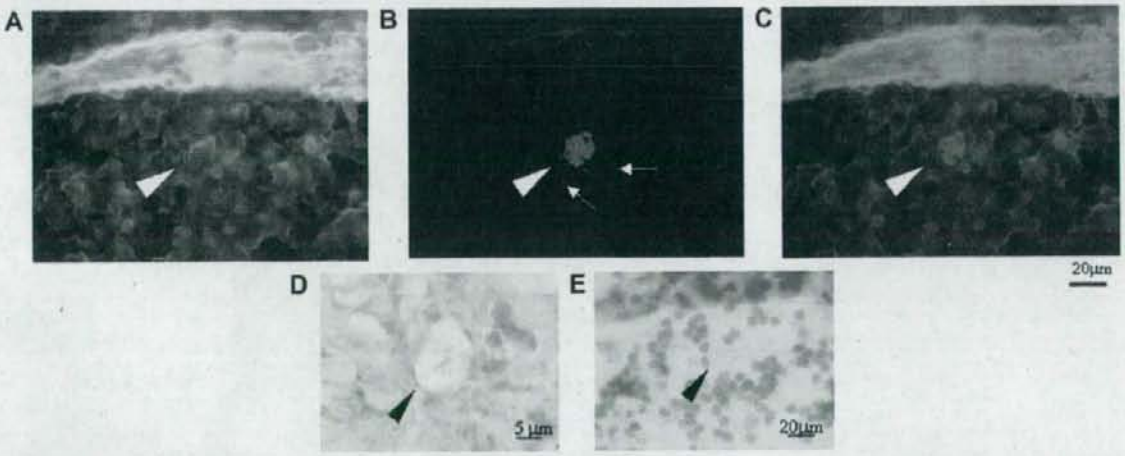

Fig. 3. Cyst containing fluorescent zoites. Serial thin sections through an embedded small piece of brain tissue containing a red fluorescent site were observed. (A–C) Direct observation of a thin section by transmitted light (A), red fluorescence (B) and an overlay of transmitted light and red fluorescence images (C). Arrowhead indicates a cyst containing red fluorescent zoites. Small arrows indicate zoites breaking out from the cyst. (D) PAS-stained thin section. Arrowhead indicates a PAS-positive cyst wall. (E) H& E-stained thin section. Arrowhead shows the cyst.

expression of the red fluorescent protein, DsRed Express (Clontech, Palo Alto, CA). The intensity of the fluorescence of the PLK/RED strain was strong enough to detect zoites inside the brain by direct whole brain observation (Figs. 1G and fig. 2A). When we confirmed the existence of red fluorescent PLK/RED zoites in brain, liver and lung tissues by observation of small chopped and crushed tissue sections on glass slides, a few single red fluorescent zoites were detected. These were detected in spots where no fluorescent zoites had been detected by whole brain observation (data not shown). These results indicate that whole brain observation is not accurate for detection of single zoites in deep tissue. However, as shown in Fig. 1G, fluorescent tachyzoites in brain tissue at the depth of 1–2 mm could be detected by this method. Despite its low sensitivity, the speed and accuracy of whole brain observation assists identification of some fluorescent zoites in the brain, and pinpoints sections for further histological analysis. In addition, whole brain observation does not require tissue to be crushed for detection of fluorescent zoites, which in turns enables histological study of fluorescent sites. Even after the plastic resin embedding procedure, red fluorescence was retained (Fig. 3). The combined use of the stage-specific expression of DsRed Express and plastic resin presents a great possibility to study the stage conversion of *T. gondii* in vivo.

Based on the immunohistological study of brain tissue samples of human immunodeficiency virus-infected patients with suspected clinical *Toxoplasma* encephalitis, it was considered that the initial site of reactivation is destroyed by tissue-destructive tachyzoites long before clinical symptoms appear (Reiter-Owona et al., 2000). In this study, we detected a reactivating cyst on the day 21 of dexamethasone treatment (Fig. 3). However, the first clinical symptoms were detected on the day 35. On the day following the onset of the first symptoms, red fluorescent tachyzoites had already disseminated into several organs (Fig. 2). These results clearly demonstrate a time-lag between initial reactivation of *T. gondii* in the brain and the appearance of clinical symptoms of *Toxoplasma* encephalitis.

It was revealed that INF-γ plays an important role in preventing the reactivation of *T. gondii* (Kang and Suzuki, 2001; Wang et al., 2004, 2005, 2007). Non-T cells and CD8-positive T cells were reported as sources of IFN-γ during chronic *T. gondii* infection which prevent reactivation (Khan et al., 1999; Wang et al., 2005, 2007). It was also reported that IL-12 is required for the maintenance of IFN-γ production of T cells during chronic *T. gondii* infection (Yap et al., 2000). The bradyzoite-specific surface antigen SRS9 is also known to be a key molecule for persistence of infections in the brain (Kim et al., 2007). However, the direct trigger of *T. gondii* reactivation in a local site of infection remains unknown. It was not previously possible to investigate or understand when reactivation occurred in a local site. In this study we were, to our knowledge for the first time, able to detect the initial site of reactivation.

It is not yet possible to provide quantitation of the frequency of reactivation events, because the number of latent cysts (9.4 ± 4.8 cysts/mouse) and detected reactivation event (only one event) is small. To discuss the frequency of reactivation events, experiments using more susceptible strains of mice and/or stronger immunosuppression is necessary. It was reported that C57BL mice presented significantly higher numbers of cysts when infected with the *T. gondii* ME49 strain than did BALB/c mice (Fux et al., 2003). It was also shown that treatment with anti-IFN-γ or anti-TNF-α initiated immediate parasite stage conversion from bradizoite to tachyzoite (Silva et al., 1998). These strains of mice and/or immunosuppressive treatments might be suitable for experiments to understand the frequency of reactivation events. However, the use of animals more resistant to *T. gondii* than mice, e.g. rats, has been proposed as better animal model relevant to human toxoplasmosis in AIDS patients (Zenner et al., 1999). To use such an animal model for the study of reactivation of *T. gondii* latent cysts in the brain, it is necessary to detect the initial site of reactivation even in the case of lower frequency of infection and/or reactivation.

Herein, we detected only one reactivating cyst in half of a brain (Fig. 3). Our study opens the way for further investigations to ascertain what happens in the microenvironment of brain tissue at the time of *T. gondii* reactivation.

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References

Bohne, W., Hunter, C.A., White, M.W., Ferguson, D.J., Gross, U., Roos, D.S., 1998. Targeted disruption of the bradyzoite-specific gene BAG1 does not prevent tissue cyst formation in *Toxoplasma gondii*. Mol. Biochem. Parasitol. 92, 291–301.

Dao, A., Soete, M., Sergent, V., Deslee, D., Fortier, B., Dubremetz, J.F., 2002. Potential of beta-galactosidase-expressing *Toxoplasma gondii* for in situ localization and observation of rare stages of the parasite life cycle. Parasitol. Res. 88, 69–72.

Djurkovic-Djakovic, O., Milenkovic, V., 2001. Murine model of drug-induced reactivation of *Toxoplasma gondii*. Acta Protozool. 40, 99–106.

Donald, R.G., Roos, D.S., 1993. Stable molecular transform ation of *Toxoplasma gondii*: a selectable dihydrofolate reductase-thymidylate synthase marker based on drug-resistance mutations in malaria. Proc. Natl. Acad. Sci. USA 90, 11703–11707.

Dzierszinski, F., Nishi, M., Ouko, L., Roos, D.S., 2004. Dynamics of *Toxoplasma gondii* differentiation. Eukaryot. Cell 3, 992–1003.

Frenkel, J.K., Escajadillo, A., 1987. Cyst rupture as a pathogenic mechanism of toxoplasmic encephalitis. Am. J. Trop. Med. Hyg. 36, 517–522.

Fux, B., Rodrigues, C.V., Portela, R.W., Silva, N.M., Su, C., Sibley, D., Vitor, R.W., Gazzinelli, R.T., 2003. Role of cytokines and major

histocompatibility complex restriction in mouse resistance to infection with a natural recombinant strain (type I-III) of *Toxoplasma gondii*. Infect. Immun. 71, 6392–6401.

Gazzinelli, R.T., Eltoum, I., Wynn, T.A., Sher, A., 1993. Acute cerebral toxoplasmosis is induced by in vivo neutralization of TNF-alpha and correlates with the down-regulated expression of inducible nitric oxide synthase and other markers of macrophage activation. J. Immunol. 151, 3672–3681.

Gazzinelli, R.T., Hartley, J.W., Fredrickson, T.N., Chattopadhyay, S.K., Sher, A., Morse, H.C. 3rd., 1992. Opportunistic infections and retrovirus-induced immunodeficiency: studies of acute and chronic infections with *Toxoplasma gondii* in mice infected with LP-BM5 murine leukemia viruses. Infect. Immun. 60, 4394–4401.

Kang, H., Suzuki, Y., 2001. Requirement of non-T cells that produce gamma interferon for prevention of reactivation of *Toxoplasma gondii* infection in the brain. Infect. Immun. 69, 2920–2927.

Khan, I.A., Green, W.R., Kasper, L.H., Green, K.A., Schwartzman, J.D., 1999. Immune CD8(+) T cells prevent reactivation of *Toxoplasma gondii* infection in the immunocompromised host. Infect Immun. 67, 5869–5876.

Kim, S.K., Karasov, A., Boothroyd, J.C., 2007. Bradyzoite-specific surface antigen SRS9 plays a role in maintaining *Toxoplasma gondii* persistence in the brain and in host control of parasite replication in the intestine. Infect. Immun. 75, 1626–1634.

Luft, B.J., Remington, J.S., 1992. Toxoplasmic encephalitis in AIDS. Clin. Infect. Dis. 15, 211–222.

Nishikawa, Y., Kawase, O., Vielemeyer, O., Suzuki, H., Joiner, K.A., Xuan, X., Nagasawa, H., 2007. *Toxoplasma gondii* infection induces apoptosis in noninfected macrophages: role of nitric oxide and other soluble factors. Parasite Immunol. 29, 375–385.

Nishikawa, Y., Xuenan, X., Makala, L., Vielemeyer, O., Joiner, K.A., Nagasawa, H., 2003. Characterisation of *Toxoplasma gondii* engineered to express mouse interferon-gamma. Int. J. Parasitol. 33, 1525–1535.

Pomeroy, C., Kline, S., Jordan, M.C., Filice, G.A., 1989. Reactivation of *Toxoplasma gondii* by cytomegalovirus disease in mice: antimicrobial activities of macrophages. J. Infect. Dis. 160, 211–305.

Reiter-Owona, I., Seitz, H., Gross, U., Sahm, M., Rockstroh, J.K., Seitz, H.M., 2000. Is stage conversion the initiating event for reactivation of *Toxoplasma gondii* in brain tissue of AIDS patients? J. Parasitol. 86, 531–536.

Silva, N.M., Gazzinelli, R.T., Silva, D.A., Ferro, E.A., Kasper, L.H., Mineo, J.R., 1998. Expression of *Toxoplasma gondii*-specific heat shock protein 70 during In vivo conversion of bradyzoites to tachyzoites. Infect. Immun. 66, 3959–3963.

Singh, U., Brewer, J.L., Boothroyd, J.C., 2002. Genetic analysis of tachyzoite to bradyzoite differentiation mutants in *Toxoplasma gondii* reveals a hierarchy of gene induction. Mol. Microbiol. 44, 721–733.

Soete, M., Fortier, B., Camus, D., Dubremetz, J.F., 1993. *Toxoplasma gondii*: kinetics of bradyzoite-tachyzoite interconversion in vitro. Exp. Parasitol. 76, 259–264.

Sumyuen, M.H., Garin, Y.J., Derouin, F., 1996. Effect of immunosuppressive drug regimens on acute and chronic murine toxoplasmosis. Parasitol. Res. 82, 681–686.

Vanchinathan, P., Brewer, J.L., Harb, O.S., Boothroyd, J.C., Singh, U., 2005. Disruption of a locus encoding a nucleolar zinc finger protein decreases tachyzoite-to-bradyzoite differentiation in *Toxoplasma gondii*. Infect. Immun. 73, 6680–6688.

Vollmer, T.L., Waldor, M.K., Steinman, L., Conley, F.K., 1987. Depletion of T-4+ lymphocytes with monoclonal antibody reactivates toxoplasmosis in the central nervous system: a model of superinfection in AIDS. J. Immunol. 138, 3737–3741.

Wang, X., Claflin, J., Kang, H., Suzuki, Y., 2005. Importance of CD8(+)Vbeta8(+) T cells in IFN-gamma-mediated prevention of toxoplasmic encephalitis in genetically resistant BALB/c mice. J. Interferon. Cytokine Res. 25, 338–344.

Wang, X., Kang, H., Kikuchi, T., Suzuki, Y., 2004. Gamma interferon production, but not perforin-mediated cytolytic activity, of T cells is required for prevention of toxoplasmic encephalitis in BALB/c mice genetically resistant to the disease. Infect. Immun. 72, 4432–4438.

Wang, X., Michie, S.A., Xu, B., Suzuki, Y., 2007. Importance of IFN-gamma-mediated expression of endothelial VCAM-1 on recruitment of CD8(+) T Cells into the brain during chronic infection with *Toxoplasma gondii*. J. Interferon. Cytokine. Res. 27, 329–338.

Yap, G., Pesin, M., Sher, A., 2000. IL-12 is required for the maintenance of IFN-gamma production in T cells mediating chronic resistance to the intracellular pathogen, *Toxoplasma gondii*. J. Immunol. 165, 628–631.

Zenner, L., Foulet, A., Caudrelier, Y., Darcy, F., Gosselin, B., Capron, A., Cesbron-Delauw, M.F., 1999. Infection with *Toxoplasma gondii* RH and Prugniaud strains in mice, rats and nude rats: kinetics of infection in blood and tissues related to pathology in acute and chronic infection. Pathol. Res. Pract. 195, 475–485.

Zhang, G.H., Vu, H.T.T., Battur, B., Zhou, J.L., Zhang, H.S., Liao, M., Kawase, O., Lee, E.G., Dautu, G., Igarashi, M., Nishikawa, Y., Zuan, X. 2007. A heterologous prime-boost vaccination regime using DNA and a vaccinia virus, both expressing GRA4, induced protective immunity against *Toxoplasma gondii* infection in mice. J. Parasitol.*in press*.